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Chlorsulfuron Inhibition of Cell Cycle Progression and the Recovery of G_1 Arrested Cells by Ile and Val

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Abstract. Cultured pea root tips were treated with 14 nM chlorsulfuron (CS) for up to 60 h. The progression of cells from G_1 into S was monitored by measuring ^{3}H-thymidine (^{3}H-Thy) incorporation into DNA. Chlorsulfuron treatment decreased the amount of ^{3}H-Thy incorporated; however, this amount never reached zero. Short-term labeling experiments indicate that the cells are arrested in a narrow band within G_1. Cell progression recovered from the CS treatment when roots were transferred to either White's medium or White's supplemented with isoleucine (Ile) and valine (Val). Lag time before recovery began in the White's or White's plus Ile and Val medium was 12 and 4 h, respectively. The initial slope of the recovery curves was similar irrespective of the type of recovery media. Increasing the duration of CS treatment did not change the length of the lag or initial slopes of the recovery curves. The level of ^{3}H-Thy incorporation decreased with increasing duration of CS treatment for root segments transferred to Ile and Val recovery medium.

Chlorsulfuron (CS) is a relatively new herbicide used for broad spectrum weed control in cereal crops (Ray 1980). The chemical combines high herbicidal activity with a rapid inhibition of cell division and plant growth. Because of the low concentration needed to express its effect, and its high specificity, CS has potential as an experimental tool to study cell cycle progression.

In excised pea roots, root growth is significantly reduced by CS concentrations as low as 2.8 nM (Ray 1984). The reduction in growth is associated with an inhibition of cell cycle progression (Ray 1982, Rost 1984). The inhibition is reversed when the amino acids isoleucine (Ile) and valine (Val) are included in the treatment media (Rost and Reynolds 1985).

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Rost (1984) showed that the cell cycle of pea root meristem cells is blocked in G_1 and G_2. Rost hypothesized that G_2 was the primary block and G_1 the secondary block. There is no data to suggest that S phase cells are directly affected. The specific location of the block within G_1 and G_2 has not been determined.

The mode of action of CS is specific, directly inhibiting the enzyme acetolactate synthase which catalyzes the first step in the biosynthesis of Ile and Val (Ray 1984). Although CS does not affect such processes as respiration, photosynthesis, and protein synthesis, the herbicide is associated with a decrease in DNA and RNA synthesis (Ray 1982, Rost 1984). Starvation for an essential amino acid is known to result in a decrease in RNA and DNA synthesis (Fowden et al. 1967). Amino acids regulate nucleic acid synthesis by either a direct or indirect mechanism.

Research reported here focuses on kinetics of the recovery of arrested G_1 cells as they resume progression into DNA synthesis (S) in cultured pea root tips. Experiments were designed to identify the specific location of the G_1 block, and the ability of previously arrested G_1 cells to recover from CS treatments of increasing duration.

Materials and Methods

Pea seeds, *Pisum sativum* L. cv. Alaska, were surface sterilized for 6 min in a 20% (v/v) commercial bleach (5.25% NaOCl) containing 0.75% (w/v) Alconox detergent (Alconox, NY, USA), then soaked for 30 s in 95% ethanol, and rinsed with sterile deionized water. Sterilized seeds were sown in moist sterile vermiculite in crystallization dishes and incubated in the dark for 4.5 days at 26°C.

Root tips (1 cm) were excised from seedlings and transferred under aseptic conditions to flasks containing 50 ml White's medium (White 1943). Flasks were placed on a rotary shaker (60 rpm) in the dark (26°C) for 24 h to allow excision effect recovery (Rost et al. 1986).

After 24 h root tips were transferred aseptically to flasks containing 50 ml treatment solution—White's medium or White's medium containing chlorsulfuron (CS). An aqueous stock solution (pH = 12) of CS was added to the medium by filter sterilization (0.22 μm) so that the final concentration was 14 nM. Chlorsulfuron treatments were stagger started so that final treatment durations (12, 24, 36, 48, and 60 h) could be transferred to recovery media simultaneously.

Following the treatment period, root segments were transferred to flasks containing 50 ml White's medium or White's medium plus a 1:1 mixture of Ile and Val, both at 50 μM. Amino acids were added to the culture medium by sterile filtration. The pH of all media was 4.8.

DNA synthesis (G_1 to S) was monitored by measuring the amount of ^{3}H-thymidine (^{3}H-Thy) incorporated during a 1 h (20 min in Fig. 3) labeling period. ^{3}H-Thy (74 GBq/mmol, 35 KBq/ml) was added to the culture flasks and the flasks then returned to the shaker. Each flask containing 10 roots (15 in Fig. 3) was considered an experimental unit for sampling. Labeled roots were re-

moved from each flask and rinsed for 30 s in White's medium containing 2 mM cold Thy. Root tips (2 mm) were excised, the tips from each flask pooled and placed in a cryogenic vial. The vials were stored in a liquid nitrogen freezer until the end of the experiment.

DNA was extracted from frozen root tips by grinding the tissue in cold 80% (v/v) ethanol and collecting the precipitate on Whatman GF/A filters on a vacuum filter apparatus. The filter was washed with 80% ethanol, dried at 70°C, placed into 10 ml Beckman Ready-Solv EP scintillation fluid, and counted in a Beckman LS 8000 scintillation counter. Experiments were repeated 2 to 4 times; a representative experiment is shown in each figure.

Results

Treating pea root segments with 14 nM CS for 12 h results in a 72% decrease in DNA synthesis (blocks G_1 to S progression) measured by ^{3}H-Thy incorporation (Fig. 1). After transfer to fresh medium containing Ile and Val, root segments displayed a 4 h lag followed by the recovery of DNA synthesis. Between 4 and 8 h after transfer, DNA synthesis increased rapidly, and by 12 h, it reached a maximum level that was almost equal to that of the control. DNA synthesis decreased slowly over the next 12 h reaching a minimum by 24 h into the recovery period. A second increase in synthesis was observed between 24 and 28 h.

Roots transferred from a 12 h CS treatment into fresh White's medium displayed a 12 h lag before DNA synthesis began to recover (Fig. 1). During the first 4 h following the lag, DNA synthesis increased rapidly and then slowed over the next 16 h.

Although the length of the lag period was different in the White's or White's plus Ile and Val transfers, the slopes of the recovery curves during the 4 h following the lag were similar.

Recovery following a 12 h CS treatment was the same for roots transferred into recovery medium even when CS was retained in the medium (Fig. 1, inset). In both treatments, recovery did not start until after a 4 h lag, and then synthesis increased rapidly during the next 8 h. Because recovery of DNA synthesis was similar whether CS was included in the recovery medium or not, in all subsequent experiments CS was excluded from the recovery medium, thus simplifying the experimental system.

^{3}H-Thymidine incorporation for roots treated with CS for 12, 24, and 36 h, and then transferred to a White's recovery medium is shown in Fig. 2A. The lag time before DNA synthesis recovered after CS treatment was 8 to 12 h. Slopes of the recovery curves for all treatment durations were approximately equal during the first 8 h following the lag. A general dip in DNA synthesis occurred after 24 h of recovery irrespective of the length of CS treatment.

DNA synthesis in roots treated with CS for 12, 24, and 36 h and then allowed to recover in White's medium plus Ile and Val is shown in Fig. 2B. Irrespective of the treatment length, recovery began within 4 h following transfer to the recovery medium. Maximum ^{3}H-Thy incorporation for the 12 and 24 h CS-treated roots occurred by 8 h after transfer. Recovery for roots treated with CS

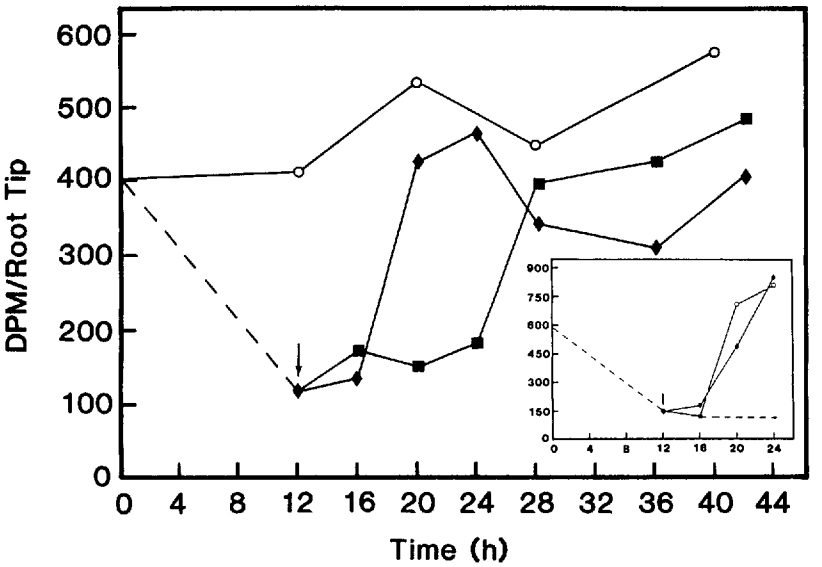

Fig. 1. ^{3}H-Thymidine incorporation in pea roots treated with 14 nM CS for 12 h (dashed line) and then transferred (arrow) to either a White's (closed squares) or White's plus Ile and Val (closed diamonds) recovery medium. The control (open circles) was a continuous exposure to White's medium. Inset: 12 h CS treatment followed by transfer to recovery medium; White's plus Ile and Val (open circles), CS plus Ile and Val (closed diamonds), or fresh CS (closed circles). Symbols indicate sampling times.

for 36 h was slower, with maximum incorporation occurring after the roots had been in the recovery media for 16 h. The maximum level of incorporation decreased with increasing CS exposure time. Slopes of the recovery curves for the 12, 24, and 36 h CS treatments demonstrated that the rate of incorporation during the first 4 h of recovery after the lag was similar irrespective of treatment duration. For the 12 and 24 h treatments, DNA synthesis continued to recover at the same rate for up to 8 h following transfer to the recovery medium.

The incorporation of ^{3}H-Thy decreased in proportion to the increasing duration of CS treatment up to 60 h (Fig. 2C). The length of the lag period before DNA synthesis began, and the initial slope of the recovery curves was approximately equal irrespective of treatment length.

Because the resolution of these experiments was limited to a 4 h minimum sampling time, another experiment was conducted to identify more specifically when DNA synthesis began to recover following a 12 h CS treatment. Following a 4.5 h lag, the rate of DNA synthesis increased quickly over the next 2 h and then declined (Fig. 3).

Discussion

Cell cycling involves the sequential progression of cells through a series of

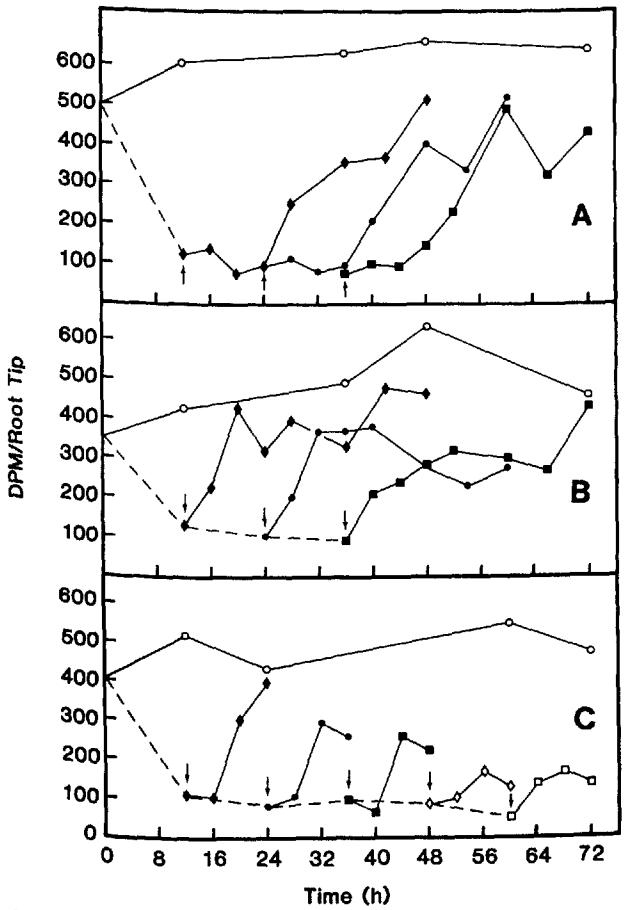

Fig. 2. ^{3}H-Thymidine incorporation in pea roots treated with 14 nM CS for up to 60 h. Treatment length was 12 (closed diamonds), 24 (closed circles), 36 (closed squares), 48 (open diamonds), or 60 h (open squares). Treated roots were transferred to either a White's (A) or White's plus Ile and Val (B,C) recovery medium. Arrows indicate when roots were transferred to recovery medium. Symbols indicate sampling times.

stages—G_1, S, G_2, M. Rost (1984) showed that CS inhibits the cell cycle by blocking cells in both G_1 and G_2. Using the incorporation of ^{3}H-Thy to monitor the progression of cells from G_1 into S, our experiments focused on the G_1 block and the ability of these cells to recover from CS treatment of increasing duration.

Treating pea root segments with CS for 12 h resulted in a significant (72%) reduction in the progression of cells from G_1 into S (Fig. 1). Cells blocked in G_1 were able to recover following a 12 h CS treatment when roots are transferred to either a White's or White's plus Ile and Val recovery medium. The pattern of recovery depended on the type of medium used.

In roots transferred to White's media, the lag before cells begin to pass from G_1 into S was 12 h, and in roots transferred to a White's plus Ile and Val

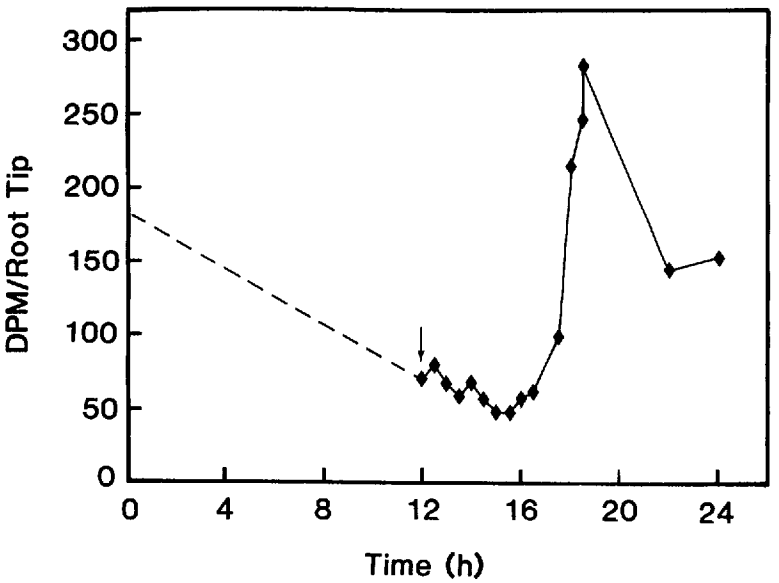

Fig. 3. ^{3}H-Thymidine incorporation in pea roots treated with 14 nM CS for 12 h and then transferred to a White's plus Ile and Val recovery medium. Arrow indicates when roots were transferred to the recovery medium. Symbols indicate sampling times.

medium, the lag was 4 h (Fig. 1). Difference in the length of the lag presumably reflects the additional amount of time necessary for the recovery of branched amino acid biosynthesis and for the newly synthesized amino acids to act.

During the first 4 h following the lag, a large number of cells enter S, and the initial rate of entry into S is the same irrespective of the type of recovery medium. After this 4 h period, the pattern of cell cycle recovery begins to differ. In an amino acid recovery medium, the number of cells entering S continued to increase over the next 4 h, but then the number declined and later increased again. In White's media, the number of cells entering S continued to increase after the first 4 h, but the rise was more gradual than observed during the first 4 h. Shapes of the 2 recovery curves can be interpreted in the following way. The steep slope of the leading edge of both recovery curves indicates that the majority of cells arrested in G_1 are highly synchronized and are blocked near a specific point. If the position of cell arrest were more general within G_1, we would expect the initial slope of the recovery curve to be less steep and the overall height to be lower.

The observed "dip," in the recovery curve following the initial steep rise was not a totally unexpected observation considering that CS blocks cell cycle progression in both G_1 and G_2 (Rost 1984) and that the population of cells arrested in G_1 are highly synchronized as we suggest. Because cell cycle recovery is more rapid in roots transferred to an amino acid rather than a White's recovery medium, the synchrony of arrested cells does not degenerate as quickly and, therefore, the "dip" represents the lag between G_1 cells entering

S and the time for cells previously arrested in G_2 to cycle through to S. When roots are transferred to White's media, the population of arrested cells has more time to become asynchronous enabling G_2 cells to blend in with the recovering G_1 cells.

Although CS is a strong inhibitor of cell cycling as measured by ^{3}H-Thy incorporation, the herbicide does not affect photosynthesis, respiration, or protein synthesis (Ray 1982). The similarity in recovery of cell cycle progression whether CS is present with Ile and Val in the recovery media (Fig. 1, inset) supports the idea that the primary mode of action of CS is the inhibition of branched amino acid biosynthesis and not the inhibition of other major plant processes.

Increasing the duration of CS treatment up to 60 h does not change the initial (first 4 h) slope of the recovery curves nor does it change the length of the lag before cell progression begins to recover for either type of recovery media (Fig. 2). The observation that the lag time does not change with increasing duration of CS treatment is contrary to the observed effect of increasing carbohydrate starvation on recovery lag time. With increasing carbohydrate starvation, length of the lag before cell progression recovers increases (Van't Hof 1970). The starvation for branched amino acids as a result of CS treatment is apparently a more specific type of metabolic effect on cell progression than is the injury caused by carbohydrate starvation.

The number of cells progressing from G_1 into S after roots have been in the amino acid recovery media for 12 h, decreased with increasing duration of CS treatment (Fig. 2C). Based on the distribution of relative DNA units, Rost (1984) suggested that the primary block for cell cycle progression in CS-treated pea roots was G_2, and that the G_1 block was a secondary, and possibly weaker block. Our observation that DNA synthesis in roots treated with CS for 12 h never reached zero (Figs. 1, 2, and 3), coupled with the observation that the mitotic index approached zero (MI $\approx$ 0.4), also supports the idea that the G_1 block is weaker and, therefore, cells arrested in G_1 tend to "leak" through over time. The observed differential sensitivity of cells blocked in G_1 and G_2 in CS-treated roots, is a similar response that cycling pea root cells have when pea roots are exposed to gamma radiation (Burholt and Van't Hof 1972).

Although the number of cells arrested in G_1 decreases with increasing duration of CS treatment, the position of the block within G_1 does not appear to change. Irrespective of treatment duration, the lag before cell progression recovered was approximately the same (Fig. 2).

Because the sample interval for experiments already discussed was so long, another experiment was conducted to resolve the pattern of cell cycle recovery. Results obtained from this experiment are consistent with those from our other CS experiments (Fig. 3). Treating pea roots for 12 h resulted in a significant decrease in the progression of cells from G_1 into S, however, progression was not completely blocked. Following transfer to the amino acid recovery medium, there was a 4.5 h lag before cell progression began to recover. Assuming little delay in the uptake of the exogenously supplied amino acids and the time for them to act, a lag of 4.5 h suggests that the position of the block is in early G_1. Once cell progression began to recover, there was a steep

increase in the number of cells moving from G_1 into S. The steep increasing slope was followed by a steep decreasing slope. The steep decreasing slope likely represents the lag between G_1 cells passing through to S and the time for arrested G_2 cells to cycle through to G_1 and S.

Results discussed in this investigation have focused on the ability and kinetics of cells arrested in G_1 to recover following CS treatment of increasing duration (up to 60 h). The mechanism by which a decrease in branched amino acid biosynthesis results in a differential block of cell progression in G_1 and G_2 is unknown. Amino acid starvation causes the so called "stringent response" in bacterial cells that is known to shut down a number of cellular processes (Fowden et al. 1967, Lewin 1985). In *E. coli,* amino acid starvation causes the accumulation of two nucleotides, guanosine tetraphosphate and guanosine pentaphosphate (Lewin 1985). Their presence is known to inhibit RNA transcription in bacteria. This response, however, has not yet been reported in higher plant cells. It is also possible that inhibition by specific amino acids causes specific transcriptional inhibition, for example, for cell cycle specific RNAs. Experiments are underway to determine if the control of cell cycle progression is specific to branched amino acids or whether this response is a general one common to other groups of amino acids as well.

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